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The effect of crystallinity on hydroxyapatite-induced production of reactive oxygen metabolites by polymorphonuclear leukocytes

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To assess the role of crystallinity in biological response, we quantitated the generation of reactive oxygen metabolites in human polymorphonuclear leukocytes (PMN) with a chemiluminescence assay using three hydroxyapatite preparations with sintering temperatures of 1,200°C and 900°C and a drying temperature of 110°C on the basis of equal weights (1 mg/ml). These crystals have almost the same average diameters and similar average zeta potentials. The crystals prepared at higher temperatures have higher crystallinity, or larger domain sizes, which were calculated by X-ray diffraction line broadening. The production of reactive oxygen metabolites by PMN in hydroxyapatite of 1,200°C was 10-times that by PMN in hydroxyapatite of 900°C and more than 50-times greater than that in hydroxyapatite of 110°C. A single linear correlation was observed for a plot of log (peak chemiluminescence levels) vs. a plot of log (domain sizes). These results clearly show that the maximal effect of crystallinity on hydroxyapatite-induced production of reactive oxygen metabolites by human PMN was seen at higher crystallinity.

Crystal; Chemiluminescence; Domain size; Sintering temperature

1. INTRODUCTION

Hydroxyapatite crystals are the inorganic constituent of osseous and dental tissues, and synthetic hydroxyapatites are interesting biomaterials. On the other hand, the deposition of hydroxyapatite microcrystals in tissues is associated with pathological syndromes. Hydroxyapatite is associated with gout-like attacks in several patients [1], is present in approximately 40% of all kidney stones [2], and is found in numerous other soft tissue calcifications [3]. The interaction between hydroxyapatite microcrystals and cells is involved in numerous aspects of both normal and pathological apatite depositions. Therefore, the study of hydroxyapatite–cell interaction is of considerable interest.

Synthetic hydroxyapatite has been prepared by a variety of sintering temperatures [4]. Jarcho [5] described hydroxyapatite fired at higher temperatures as showing increasing grain growth and opacity, although the composition determined by X-ray diffraction was still 100% hydroxyapatite. The various sintering temperatures of hydroxyapatite provide a unique opportunity to study cell interactions with crystals having different crystallinity while maintaining a nearly constant lattice. Microcrystals placed in the body will stimulate phagocytic cells and induce host reactions [6,7]. Polymorphonuclear leukocytes (PMN) are important phagocytic cells in the initial non-specific reaction. Reactive oxygen metabolites formed by phagocytic cells have biological effects on the development of host reactions, e.g. damage to DNA [8], inactivation of alpha-1-antiprotease enzyme-binding capacity [9], and bioactivation of chemical carcinogens [10]. Therefore, it is very important to study the interaction between different crystallinities of hydroxyapatite and PMN. In this study we determined the effect of crystallinity on the generation of reactive oxygen metabolites in human PMN with a chemiluminescence assay using three hydroxyapatite preparations with different sintering temperatures in order to better define the role of crystallinity in crystal–cell interactions.

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Abbreviations: PMN, polymorphonuclear leukocyte; PBS, Dulbecco's phosphate buffered saline; MEM, Dulbecco's modified Eagle's medium.

2. MATERIALS AND METHODS

2.1. *Materials*

For this study, three types of hydroxyapatite were prepared by Mitsubishi Materials Co., Tokyo, Japan. They were prepared using a slow precipitation method involving aqueous $Ca(OH)_2$ and H_3PO_4 at 40°C and careful digestion and extraction procedures. Then they were sintered at 1,200°C and 900°C, or dried at 110°C without sintering, respectively. The crystals were ground down to the desired particle size. The protein content of these particles was checked by the direct Ninhydrin reaction and Bradford's method using Coomassie brilliant blue [11]. No trace of protein appeared. The crystals were verified to be pyrogen-free by the limulus amebocyte lysate assay (Sigma Co., St. Louis, MO) following FDA guidelines [12]. These particles were

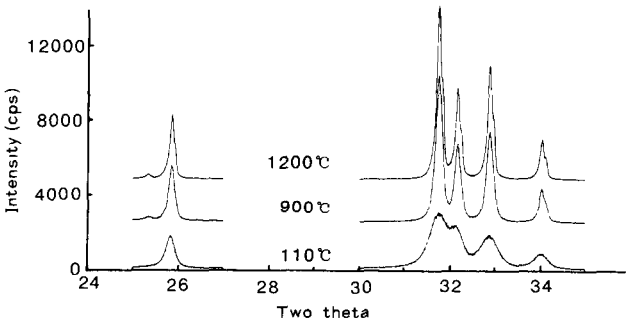

Fig. 1. X-ray diffraction patterns of the three hydroxyapatite particles measured by Model RU-200.

round. We have designated the three types of hydroxyapatite as 1,200°C, 900°C, and 110°C.

2.2. *X-ray analysis and line broadening*

Confirmation of crystal structure and purity was obtained by high resolution X-ray powder diffraction with monochromatic CuKa radiation on a rotating anode X-ray generator (Rigaku, Model RU-200 and RINT-2400, Tokyo, Japan). Measurements by Model RU-200 were made using the step-scanning technique (40 kV, 100 mA; 4°/min) in the range of 2θ=5–60° at room temperature. Patterns were compared to a known hydroxyapatite standard. Measurements by Model RINT-2400 were made using the step-scanning technique (40 kV, 100 mA; 0.5°/min) in the range of 2θ=25–27° for the (002) and in the range of 2θ=30–35° for the (300) at room temperature. Following standard methods [13], the peak width at half height was measured for (002) and (300) diffraction maxima from densitometer tracings Domain sizes were calculated using the Scherrer equation [13]. The instrumental line broadening was determined to be 0.105° 2θ using a sample of quartz. The size of the quartz crystals was determined to be 36.2 ± 13 μm by direct measurement of particles in a scanning micrograph (Model JSM-T20; JEOL Ltd., Tokyo, Japan). The X-ray diffraction patterns of this series of synthetic hydroxyapatites showed sharpening of the apatite peaks with increased sample heating temperatures (Fig. 1).

2.3. *Infrared spectroscopy*

Infrared spectra were made of all specimens on an Infrared spectrum's analyzer (Shimadzu, Model FTIR-4000, Kyoto, Japan) operating in the absorbance mode. KBr pellets were used in order to facilitate quantitation of the results. All specimens exhibited apparently symmetric 630–640 cm^{-1} OH^- stretch peaks. In the samples prepared at high temperatures, the OH^- stretch band exhibited pronounced asymmetry (toe extending toward lower frequencies), similar to that discussed by Cant et al. [14] in spectra taken at elevated temperatures [4].

2.4. *Particle measurements*

Crystal sizes were determined using a particle size analyzer (Model SALD-1100; Shimadzu, Kyoto, Japan). The surface areas were measured with a surface area analyzer (Model Monosorb. MS-13; Yuasa-Ionics Corp., Osaka, Japan). One-point Brunauer–Emmett–Teller (BET) surface area determinations were made using nitrogen (adsorbate) in helium (carrier) gas mixtures. Fig. 2 shows the crystal size distribution, average crystal diameter, and specific surface area for each of the three types of hydroxyapatite used in this study These crystals have almost the same average diameters of 3.0, 3.2 and 3.4 μm, respectively. About 3 μm was chosen because our previous study showed that the 3.2-μm alumina fraction demonstrated the highest induction of reactive oxygen metabolites in PMN and mononuclear leukocytes when we quantitated the generation with a chemiluminescence assay using 6 alpha-alumina preparations with average diameters of 0.6, 0.8, 3.2, 7.5, 28 and 68 μm on an equal weight (1 mg/ml) and surface area (100 cm^2/ml) basis [15]. Our preliminary study showed that in each of the three hydroxyapatite preparations about 3 μm of the hydroxyapatite fraction demonstrated the highest induction of reactive oxygen metabolites in PMN when we quantitated the generation with a chemiluminescence assay using 16 hydroxyapatite preparations with average diameters of 1.0, 1.6, 3.0, 5.4, 7.2 and 9.5 μm in 1,200°C, 1.0, 1.6, 3.2, 5.4, 7.2 and 9.2 μm in 900°C, and 3.4, 5.1, 7.1 and 8.7 μm in 110°C on an equal weight (1 mg/ml) basis. The zeta potential (a measure of the crystal surface charge) was determined using a zeta potential analyzer (System-3000; Penkem) for each of the hydroxyapatite samples suspended in distilled water. These crystals have similar average zeta potentials of −12.2, −8.1 and −13.2 mV in 1,200°C, 900°C and 110°C, respectively.

2.5. *PMN isolation*

PMN were isolated by Ficoll-Hypaque density centrifugation from heparinized human venous blood, obtained from apparently healthy adults. PMN were removed separately, washed in Dulbecco's phosphate buffered saline (PBS), and counted More than 98% of the cells were PMN, as assessed from Wright–Gimsa stained smears, and the level of cell viability exceeded 99% as determined by Trypan blue dye exclusion.

2.6. *Chemiluminescence assay*

Chemiluminescence was measured with an automatic microcomputer-controlled luminescence analyzer (LB 9505 AT; Berthold, Germany) at 37°C. The reaction mixture (final total volume 1.0 ml) consisted of 20–40 μl of leukocyte suspension (final concentration 5×10^5 cells/ml); 20 μl of 10^{-4} M luminol (5-amino, 2,3-dihydro, 1,4-phthalazine-dione) (Tokyo Kasei Kogyo, Tokyo, Japan) in Dulbecco's modified Eagle's medium (MEM); and 100 μl of hydroxyapatite particles suspended in PBS (final concentration 1 mg/ml). The chemiluminescence output was monitored for 20 min after particles were added to the cells. Results were expressed as counts per minute (cpm) per 5×10^5 cells. Cell-free reaction mixtures with hydroxyapatite caused no CL responses.

2.7. *Statistical analysis*

Data points indicated the means ± S.D. One factor analysis of variance (ANOVA) with repeated measures was used to evaluate the effect of crystallinity on hydroxyapatite-induced production of reactive oxygen metabolites by PMN. Differences between treatment means were determined by the Scheffe procedure. The 0.05 level was used as the criterion of statistical significance. Coefficients of correlation were calculated by standard statistical methods.

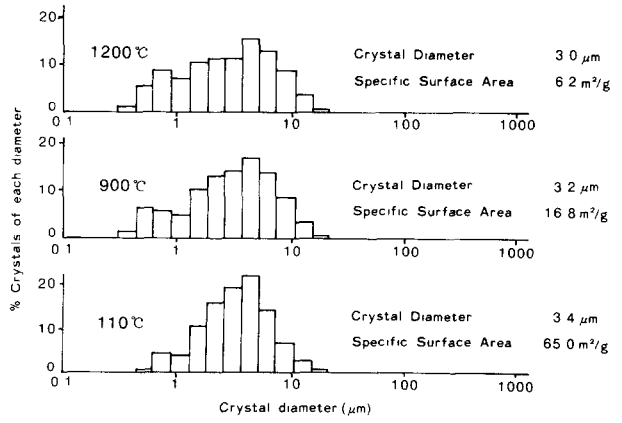

Fig. 2. Crystal size distribution of the three hydroxyapatite particles used in this study. The average crystal diameter and specific surface area are included for each particle.

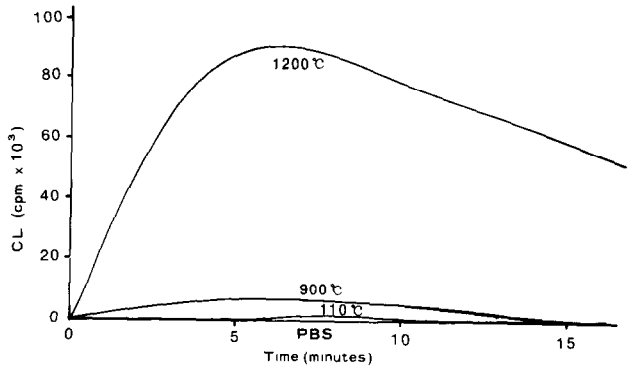

Fig. 3. Kinetics of chemiluminescence of polymorphonuclear leukocytes (PMN) induced by hydroxyapatite particles with sintering temperature of 1,200°C and 900°C and with drying temperature of 110°C, and Dulbecco's phosphate buffer saline (PBS).

3. RESULTS

Fig. 3 shows the kinetics of chemiluminescence of PMN induced by hydroxyapatite particles with sintering temperatures of 1,200°C and 900°C or a drying temperature of 110°C on an equal weight basis. Hydroxyapatite particles induced PMN chemiluminescence in the following order of magnitude: 1,200°C > 900°C > 110°C > PBS. Peak chemiluminescence levels were reached at 6.1, 5.6, 6.9 and 7.2 min in 1,200°C, 900°C, 110°C and PBS, respectively. The production of reactive oxygen metabolites by PMN in hydroxyapatite of 1,200°C was 10-times that by PMN in hydroxyapatite of 900°C, and more than 50-times greater than that in hydroxyapatite of 110°C. One factor ANOVA with repeated measures showed that significant differences were present among 1,200°C, 900°C, 110°C and PBS, except between 110°C and PBS.

Fig. 4 shows the correlation between peak chemiluminescence level and the domain size calculated from the line broadening of the (300) and (002) diffraction maxima for each crystal. After removing the component of the line broadening which was due to the instrument, no attempt was made to separate the experimentally determined line broadening into the components due to domain size and atomic substitutions. This may explain some of the scatter in the domain size vs. chemiluminescence response. Although line broadening analysis is more accurate when used to determine domain sizes less than 1,000 Å [4], a single linear correlation is observed for a plot of log (peak chemiluminescence levels) vs. a plot of log (domain sizes) for both the (300) and (002). The coefficient for the (300) was $r = 0.997$, $P < 0.05$, while that for the (002) was $r = 1.000$, $P < 0.01$.

4. DISCUSSION

It has been demonstrated that host defense reactions to particles by phagocytic cells are largely affected by size and the surface properties of the particles, especially the chemical structure of the surface and surface charge [16–19]. In the present study we found that the crystals prepared at lower temperatures, which have more surface area and almost the same average diameter and similar average zeta potentials, induced less chemiluminescence than the others. The difference in chemiluminescence was apparently due to a difference in sintering temperature, or a difference in the crystallinity of the hydroxyapatite. A study of the hemolytic potential induced by hydroxyapatite suggests that the crystal–cell interactions may be a crystallinity-related phenomenon [4], which was also seen in the case of monosodium urate monohydrate [20]. Wiessner et al. [21] compared four different silicon dioxide and two different titanium dioxide crystals. Similar to our results, they demonstrated that biological activity seemed to correlate with percent occupied volumes which were calculated using known crystallographic data for each of the crystals. Hydroxyapatite is an anisotropic structure with hexagonal symmetry [22]. The six-fold rotation axis is the crystallographic z-axis and is related to the (002) diffraction maxima. The diffraction maxyima (300) is related to other structural features of hydroxyapatite lattice that do not experience the degree of long-range order observed along the (002) [4]. It is for these reasons that the correlations between domain size and chemiluminescence for these sets of maxima are different. Although some studies showed no difference in bonding between hydroxyapatite and bone tissue at different sintering temperatures [23], the greater phlogistic potential of higher crystallinity of crystals or ceramics in clinical use needs to be taken into consideration. Our findings provide further evidence that the qualitative nature of crystallinity may play a controlling role in phagocytic cell function.

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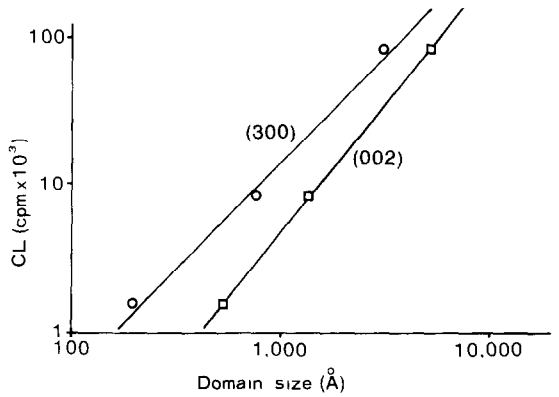

Fig. 4. Plot of the domain size calculated from the line broadening of the (300) (○) and (002) (□) diffraction maxima for each crystal vs. peak chemiluminescence level.

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